Histone Changes During Degeneration and Regeneration of the Pancreas

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1. Changes in the microstructure of histones from the pancreas were followed in rats given ethionine while on a protein-free diet. The lysine-rich histone F1 and the other histones extracted subsequently with 250 mM-hydrochloric acid were isolated. When the ethionine-treated rats returned to the stock diet, regeneration of the pancreas was accompanied by an immediate increase in phosphate content of the total histone extract from which histone F1 had been removed. After 3-4 days there was a further increase in the phosphate content of this extract, and also in that of histone F1. 2. Similar changes in the phosphate and thiol+disulphide content of pancreas histones other than F1 were produced by starvation and re-feeding.

In the rat after partial hepatectomy the onset of liver regeneration is associated with an increased phosphate content of the total histone extract from which the lysine-rich histone, F1, has been removed. The histone(s) whose seryl residues carry this increased content of phosphate are not yet defined but the increased phosphorylation coincides with the onset of new RNA synthesis. There is also an increased phosphorylation of the total histone extract from which F1 has been removed, and of the histone F1, during the period of DNA synthesis (Ord & Stocken, 1968, 1969).

Ethionine administration to rats on a protein-free diet causes profound degeneration in the acinar cells of the pancreas in 7-10 days. If the injections are stopped and the animals transferred to a normal stock diet, repair and regeneration occur (Fitzgerald *et al.* 1968a). Increased RNA synthesis and the re-formation of pancreatic enzymes takes place during the regenerative phase (Marsh, Goldsmith, Crocco & Fitzgerald, 1968). DNA synthesis in the acinar cells is increased markedly from the second day on the stock diet, reaching a peak after 4 days of re-feeding (Fitzgerald, Carol & Rosenstock, 1966; Fitzgerald, Vinijchaikul, Carol & Rosenstock, 1968b). The pancreas therefore offered a second mammalian model in which to investigate the association of changes in different histones with events leading to and occurring during the increased RNA and DNA synthesis characteristic of regeneration.

METHODS

Animals. Males (180-220 g) of the Oxford Laboratory's strain of Wistar rats were used; the animals and diets at the Downstate Centre were as described previously (Fitzgerald *et al.* 1968a). The stock diet in Oxford was the Oxoid breeding diet (Oxoid Ltd., Southwark Bridge Road, London S.E.1, U.K.). The protein-free diet used during ethionine administration was similar to the protein-free diet used earlier (Fitzgerald *et al.* 1968a). DL-Ethionine (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) was injected intraperitoneally (0.7 g/kg body wt.) daily in 5-8 ml of water for 5 days. From the fifth day to the seventh or eighth day of administration the dose was decreased daily by about 15%. The mortality for the animals receiving ethionine in the Oxford group was between 30 and 40%. In the Downstate group the mortality was about 15% and the full dose was given for 7 days.

The animals were injected for 7 or 8 days and were returned to the stock diet on the eighth or ninth day. Control animals were kept on the stock diet throughout. All experimental animals were housed in individual cages with water available *ad lib*.

Nuclei. Nuclei from the pancreas were prepared in 0.25 M-sucrose-5 mM-MgCl₂-5 mM-tris-HCl, pH 7.1, by the method described by Creasey & Stocken (1959) for thymus nuclei. The sedimented nuclei were washed once in 0.25 M-sucrose-5 mM-MgCl₂-5 mM-tris-HCl, pH 7.2, twice with 10 mM-tris-HCl-5 mM-MgCl₂, pH 7.1, and once with 1 mM-HCl. Each preparation was examined by phase-contrast microscopy and a few pellets of nuclei were examined by electron microscopy.

Relatively clean nuclei, free from zymogen granules and other cellular organelles, were obtained.

Isolation of histones. The histones were obtained as described by Ord & Stocken (1969). The nuclei were extracted twice with 5% (w/v) trichloroacetic acid, and histone F1 was precipitated by the addition of trichloroacetic acid to 20% (w/v). This precipitate was redissolved in 5% (w/v) HClO₄, reprecipitated with trichloroacetic acid, dissolved in 250mm-HCl, and dialysed against 50mm-HCl and water at 0°C. Analyses of histone F1 prepared in this way agreed with those previously reported (Johns, 1964; R. H. Buckingham & L. A. Stocken, unpublished work). The histones from which histone F1 has been removed (Ord & Stocken, 1969) were extracted from the residual nuclei by 250mm-HCl and this extract was also dialysed as above. Amino acid analyses of extracts prepared in Oxford and at the Downstate Centre agree. There was no difference in composition between extracts obtained from starved rats and from starved rats returned to the stock diet for 1 day.

[³H]Thymidine uptake into DNA. The rats received 0.25 µCi of [³H]thymidine/g body wt. (specific radioactivity 5 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) intraperitoneally 1 h before death. Two sections of pancreas were taken from each animal for radioautograms and 10000–50000 nuclei were counted to determine the percentage of nuclei labelled (Fitzgerald *et al.* 1968a).

[³H]Uridine uptake into RNA. The tissue was homogenized (Marsh *et al.* 1968) and sodium dodecyl sulphate added to a final concentration of 0.1% (w/v). The suspension was precipitated at a final concentration of 5% (w/v) trichloroacetic acid and washed once. Trichloroacetic acid and lipid were removed by ether-ethanol (3:1, v/v) washes, the residue was dissolved at pH 12 and samples were taken for measurement of ³H incorporation.

The radioactivity was determined in a Nuclear-Chicago liquid-scintillation counter. Counting efficiency was 25% and sufficient counts were recorded to give an accuracy of ±5%.

Electrophoresis. Analytical electrophoresis on polyacrylamide gel was performed as described by McAllister, Wan & Irvin (1963) with glycine-acetic acid buffer, pH 4.0, at 300 V and 4–5 mA/tube.

Methods for the determination of protein, thiol, thiol+disulphide and phosphate contents of the histones are as described earlier (Ord & Stocken, 1968). In the experiments carried out at the Downstate Centre, thiol+disulphide contents were not measured. The phosphate contents are therefore expressed as absolute values, and also as percentages of the control values so that the results from Downstate and Oxford may be directly compared.

RESULTS

The weight losses of the rats and of their pancreases and the decrease of DNA synthesis were similar for equal time-periods to those reported earlier for a longer ethionine regimen (Fitzgerald *et al.* 1968a). When the ethionine injections were stopped and the animals were returned to the stock diet, the rats began to gain total body and pancreas weight rapidly (Table 1). Thymidine uptake into DNA of the pancreas began to increase after 2 days of re-feeding with the stock diet (Table 1) and

Table 1. *Changes in DNA and RNA synthesis and in histones from pancreas nuclei obtained from rats given ethionine on a protein-free diet*

Day of treatment	Days back on stock diet	Wet wt. of pancreas (g)*†	Thymidine uptake as % of labelled nuclei*	Specific radio-activity of RNA as % of control*	Phosphate content of histones as % of control*†		Phosphate and thiol contents of total histones from which F1 had been removed† (nmol/mg of protein)	
					Histone F1	Total histone-F1	Phosphate	Thiol+disulphide
0	—	0.96 (10)	0.46±0.03	100	100	100	74.4±17.3 (9)	75.0±19.5 (9)
8	0	0.40 (3)	0.11±0.01	43 (1)	74 (5)	74 (5)	48.9 (2)	45.0 (2)
9	1	0.41 (3)	0.18±0.06	30 (1)	81 (5)	92 (6)	64.2 (3)	36.0 (3)
10	2	0.41 (3)	0.99±0.31	78 (1)	103 (3)	97 (3)	—	—
11	3	0.51 (3)	5.77±1.18	137 (1)	152 (6)	98 (6)	74.0 (3)	60.2 (3)
12	4	0.52 (3)	10.38±2.54	170 (1)	144 (3)	107 (3)	—	—
15	7	0.60 (3)	1.70±0.36	120 (1)	118 (3)	105 (3)	—	—
23	15	0.93 (2)	0.54±0.11	—	97 (4)	107 (5)	71.6 (2)	70.0 (2)

* Results obtained in the Downstate Centre.

† Results obtained in Oxford.

reached a peak on day 12 after 4 days back on the stock diet. After 15 days back on the stock diet, the total body weight of the experimental animals had reached about 85% of the weight of the controls, the pre-experimental pancreas weight had almost been regained and the rate of DNA synthesis had returned to normal.

The total RNA content of the pancreas was decreased by about 40% after ethionine administration for 7 days; uridine uptake was decreased by about 60% (Table 1). After 2 days of re-feeding, RNA synthesis increased and reached a peak at day 12.

The phosphate content of histone F1 decreased after 7 days of ethionine administration (Table 1). Little alteration in this phosphorylation was produced after 1 day back on the stock diet but an increased phosphate content of histone F1 was found after 3–4 days back on the normal diet.

Changes also occurred in the total histones extracted after removing histone F1 (Table 1). Both the thiol+disulphide and phosphate concentrations were higher than those reported earlier in liver (Ord & Stocken, 1969) although the ratio of thiol+disulphide to phosphate (1:1) was the same. Ethionine administration caused a fall in both the thiol+disulphide and phosphate contents of the extract, the ratio of the two remaining roughly constant. When the rats were re-fed with the stock diet for 1 day the phosphate content increased, without any increase in thiol+disulphide. After 3 days of re-feeding with the stock diet the phosphate content remained elevated over the minimal value, whereas the thiol+disulphide con-

tent increased above the minimal value but was still below that of the controls.

Because the ethionine-treated animals ate very little, an additional group of starved rats was used (Table 2). By the eighth day the surviving starved rats had lost as much body weight as the ethionine-treated animals and their pancreas weights were almost as low. Very similar changes were found in the histones from which F1 had been removed (Table 2) to those following ethionine administration to rats on a protein-free diet. The sulphur and phosphate contents of the extract decreased, the ratio of the two remaining constant. One day back on the stock diet again caused an increased phosphorylation of histones other than F1, with no change in the amino acid content of the extracts. In contrast with rats recovering from ethionine treatment phosphorylation of histone F1 increased to only about control values. The radioautograms of the starved-re-fed animals showed a decreased thymidine incorporation during the 7 days of starvation and there was a slow increase to control values at days 12 and 15 (Table 2).

DISCUSSION

Changes in the pancreas caused by return to the stock diet after ethionine administration are very similar to those produced in liver regenerating after partial hepatectomy. There is an increase in RNA synthesis followed by a wave of DNA synthesis; organ weight increases more slowly. Changes in liver produced by starvation are well known (Harrison, 1953); protein and RNA are lost but the

Table 2. *Changes in DNA synthesis and in histones from pancreas nuclei obtained from starved and re-fed rats*

The treatment of the rats and the methods of analysis are given in the text. The number of experiments is given in parentheses; 10–22 rats were used/experiment; in the radioautography four rats were examined individually for each point.

Day of treatment	Days back on stock diet	Wt. of pancreas (g)*†	Thymidine uptake as % of labelled nuclei*	Phosphate content of histones as % of control*		Phosphate and thiol content of total histones from which F1 had been removed† (nmol/mg of protein)	
				Histone F1	Total histone – F1		
0	—	0.84	0.46±0.03	100	100	74.4±17.3	75.0±19.5
8	0	0.40 (3)	0.01±0.01	72 (3)	107 (3)	41.3 (2)	38.4 (3)
9	1	0.46 (5)	0.01±0.01	69 (3)	120 (3)	60.3 (2)	44.9 (2)
10	2	0.54 (3)	0.05±0.01	90 (3)	128 (3)	—	—
11	3	0.57 (3)	0.15±0.04	85 (3)	117 (3)	—	—
12	4	0.62 (3)	0.28±0.13	102 (3)	102 (3)	—	—
15	7	0.80 (3)	0.33±0.13	79 (3)	111 (3)	—	—

* Results obtained in the Downstate Centre.

† Results obtained in Oxford.

DNA content/cell is unaltered. In the pancreas the pattern seems similar so that although the weight loss caused by starvation is virtually identical with that caused by the ethionine regimen, re-feeding after the ethionine treatment provokes a marked wave of DNA synthesis which is missing when growth recommences after starvation.

Changes in phosphate content of the histones also resemble those found earlier in liver regeneration (Ord & Stocken, 1969). Interpretation of the results depends critically on the nature of the protein analysed. Kleinsmith, Allfrey & Mirsky (1966) have described an increase in [^{32}P]phosphate incorporation into non-histone protein in nuclei from phytohaemagglutinin-stimulated lymphocyte cultures during the period of increased RNA synthesis. Net phosphorylation of histone F1 in such preparations is not increased until DNA synthesis commences some 40h after phytohaemagglutinin addition (M. E. Cross & M. G. Ord, unpublished work). Amino acid analyses of our histone F1 preparations agree with those given by other workers (Johns, 1964). Moreover R. H. Buckingham & L. A. Stocken (unpublished work) have shown that with highly phosphorylated histone F1 obtained from regenerating liver or ascites-tumour cells (Stevely & Stocken, 1968) the composition of phosphorylated F1 obtained by preparative electrophoresis has the same amino acid content as that in the literature and the peptide 'maps' are virtually identical with those from non-phosphorylated histone F1.

The histones extracted with 250mM-hydrochloric acid are unlikely to contain loosely bound acidic protein, since if any were present in the washed nuclei they would most probably have been removed by the extraction with 5% trichloroacetic acid. Electrophoresis of the histone extract obtained after the removal of F1 showed no protein staining on the top of the gels or just entering the gels; the pattern of components indicated the presence of histones F2A, F2B and F3. Phosphorylated peptides from those histones and from F1

have been described (Dixon, Marushige, Ling, Sung & Wigle, 1969).

The histones extracted after removal of F1 show differences between results obtained at Downstate and at Oxford which may be related to the greater mortality shown by the Oxford rats. Nevertheless the pattern of phosphate changes is very similar and strengthened the suggestion that phosphorylation of histones other than F1 precedes the phosphorylation of histone F1, and may be linked with new RNA synthesis.

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REFERENCES

- Creasey, W. A. & Stocken, L. A. (1959). *Biochem. J.* **72**, 519.
- Dixon, G. H., Marushige, K., Ling, V., Sung, M. & Wigle, D. T. (1969). *Fedn Proc. Fedn Am. Soc. exp. Biol.* **28**, 1898.
- Fitzgerald, P. J., Carol, B. & Rosenstock, L. (1966). *Nature, Lond.*, **212**, 594.
- Fitzgerald, P. J., Herman, L., Carol, B., Roque, A., Marsh, W. H., Rosenstock, L., Richards, C. & Perl, D. (1968a). *Am. J. Path.* **52**, 983.
- Fitzgerald, P. J., Vinijchaikul, K., Carol, B. & Rosenstock, L. (1968b). *Am. J. Path.* **52**, 1039.
- Harrison, M. F. (1953). *Biochem. J.* **55**, 204.
- Johns, E. W. (1964). *Biochem. J.* **92**, 55.
- Kleinsmith, L. J., Allfrey, V. G. & Mirsky, A. E. (1966). *Science, N.Y.* **154**, 780.
- McAllister, M. C., Wan, Y. C. & Irvin, J. L. (1963). *Analyt. Biochem.* **5**, 321.
- Marsh, W. H., Goldsmith, S., Crocco, J. & Fitzgerald, P. J. (1968). *Am. J. Path.* **52**, 1013.
- Ord, M. G. & Stocken, L. A. (1968). *Biochem. J.* **107**, 403.
- Ord, M. G. & Stocken, L. A. (1969). *Biochem. J.* **112**, 81.
- Stevely, W. S. & Stocken, L. A. (1968). *Biochem. J.* **110**, 187.